VIROLOGY 249, 316–324 (1998)
ARTICLE NO. VY989346

Neutralization of Different Geographic Strains of the Hepatitis E Virus with Anti-Hepatitis E Virus-Positive Serum Samples Obtained from Different Sources

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Received March 27, 1998; returned to author for revision May 21, 1998; accepted July 22, 1998

A recently developed polymerase chain reaction (PCR)-based cell culture neutralization assay was used to investigate cross-neutralization of known hepatitis E virus (HEV) strains obtained from various HEV-endemic regions of the world with different anti-HEV-positive serum samples. Serum specimens obtained from cynomolgus macaques experimentally infected with strains from Burma, Mexico, or Pakistan cross-neutralized the infectivity of each strain as well as an isolate from Morocco. Serum samples obtained either from infected patients who reside in HEV-endemic regions of the world or from U.S. residents who became infected while traveling to such regions also neutralized all four strains. In contrast, antibodies obtained from rabbits immunized with full-length Burma strain ORF2 protein neutralized only the Burma and Pakistan strains, not the Mexico or Morocco strains. In addition, antibodies obtained from guinea pigs immunized with an N-terminal truncated Burma strain ORF2 protein neutralized each strain except the Morocco strain. These data strongly suggest that antibodies elicited during an HEV infection demonstrate broad HEV neutralizing activity, whereas antibodies elicited after immunization with recombinant Burma ORF2 protein demonstrate a more limited ability to neutralize various HEV strains obtained from different regions of the world endemic for the disease.

INTRODUCTION

The hepatitis E virus (HEV) causes acute viral hepatitis worldwide; the highest incidence occurs within developing countries (Fields *et al.,* 1993; Purcell, 1996). The disease is transmitted mainly by the fecal-oral route, often through contaminated water, and occurs in both epidemic and sporadic forms (Balayan *et al.,* 1983). HEV infection appears to account for >50% of acute viral hepatitis observed among young to middle-aged adults (Datta *et al.,* 1987) and is associated with an unusually high mortality rate, up to 20%, in infected pregnant women (Purcell and Ticehurst, 1988). Sporadic cases have also been identified in developed countries, primarily as a result of travelers who import the disease from highly disease-endemic regions of the world (Centers for Disease Control and Prevention, 1993). In regions of low endemicity, such as the United States and Western Europe, the seroprevalence of antibodies to HEV (anti-HEV) among normal blood donor populations has been determined to be 2–7% (Dawson *et al.,* 1992).

HEV is an icosahedral, nonenveloped virus that is 27–34 nm in diameter with a single-strand, positive-sense, polyadenylated RNA genome of ~7.5 kb in length (Bradley *et al.,* 1987; Reyes *et al.,* 1990). The HEV genome contains three open reading frames (ORF1, ORF2, and ORF3). ORF1, which encodes for nonstructural proteins, is the largest of the ORFs and is located within the 5′-terminal part of the genome. ORF2, located within the 3′-terminal part of the HEV genome, encodes capsid protein. The smallest ORF, ORF3, overlaps ORF1 and ORF2 and encodes an immunogenic protein of unknown function (Tam *et al.,* 1991).

The entire genome of several HEV strains from Asia and North America (Mexico) has been sequenced (Tam *et al.,* 1991; Tsarev *et al.,* 1992; Aye *et al.,* 1992a, 1993; Huang *et al.,* 1992; Bi *et al.,* 1993; Yin *et al.,* 1994; Donati *et al.,* 1997). For some other strains, only partial sequences of the genome were identified (Aye *et al.,* 1992b; Yin *et al.,* 1993; Huang *et al.,* 1995; Panda *et al.,* 1995; Drabick *et al.,* 1997; Gouvea *et al.,* 1997). A comparison of nucleotide sequences of different geographic HEV strains revealed significant differences between the Asian strains and the Mexican strain. The Asian strains, although very similar to each other, can be subdivided into two subgroups: Southeast Asia including Burma, Nepal, and parts of India, and Northern and Central Asia, including Pakistan, China, Kirgizia, and India (Tsarev *et al.,* 1992; Yin *et al.,* 1994; Gouvea *et al.,* 1997). Recently, the genomes of several HEV strains from Africa were partially sequenced (Chatterjee *et al.,* 1997; van Cuyck-

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0042-6822/98 $25.00

Gandré *et al.,* 1997). Although quite distinct from all known Asian strains, the African strains were more similar to the Asian strains than to the Mexican strain. These data suggest that the genetic heterogeneities of HEV strains are geographically distributed and appear to fall into four groups: Southeast Asian, Northern and Central Asian, North American, and African (Yin *et al.,* 1994; Purcell, 1996; Chatterjee *et al.,* 1997; van Cuyck-Gandré *et al.,* 1997). More recently, an HEV strain isolated from swine and another strain isolated from a patient with acute hepatitis in the United States were grouped into a new genotype of HEV (Meng *et al.,* 1997b; Schlauder *et al.,* 1998).

Despite these observations in sequence heterogeneity, no significant serological differences were found between different HEV strains by immunoelectron microscopy and immunofluorescent assay (Bradley *et al.,* 1988; Bradley, 1990; Krawczynski *et al.,* 1991). All diagnostic tests based on recombinant HEV antigens (Yarbough *et al.,* 1991; Dawson *et al.,* 1992; Goldsmith *et al.,* 1992; Lok *et al.,* 1992; He *et al.,* 1993; Tsarev *et al.,* 1993), synthetic peptides (Dawson *et al.,* 1992; Khudyakov *et al.,* 1993; Favorov *et al.,* 1994), or a mosaic HEV protein (Favorov *et al.,* 1996; Fields *et al.,* 1996) can detect anti-HEV activity in serum specimens from patients or primates experimentally infected with HEV strains from different regions of the world. Only the C-terminal region of the ORF3-encoded protein demonstrated strain-specific antigenic reactivity. A short fragment of the ORF3 protein derived from the HEV Burma strain did not immunoreact with serum samples from cynomolgus macaques (cynos) experimentally infected with the Mexico strain. Conversely, a recombinant protein derived from the Mexico strain did not immunoreact with serum specimens from cynos infected with the Burma strain (Yarbough *et al.,* 1991). This strain specificity was also observed with several synthetic peptides derived from the ORF3 protein (Khudyakov *et al.,* 1993, 1994a; Coursaget *et al.,* 1996).

In contrast, the heterogeneity of the HEV neutralization immune response has not been thoroughly investigated. Preliminary observations showed that cynos infected with the Burma strain were immune to reinfection by the Mexico strain (Bradley, 1990) and rhesus monkeys infected with HEV from the former USSR were also immune to subsequent challenge with an India strain (Arankalle *et al.,* 1993). In addition, the immunity elicited during infection with one Indian HEV isolate protected against different Indian HEV isolates (Arankalle *et al.,* 1995). These observations suggest that neutralizing anti-HEV antibodies are protective against reinfection with either the homologous or heterologous strains. However, only one of two cynos immunized with a recombinant HEV Burma strain ORF2 protein showed full protection against challenge with the homologous Burma strain, whereas another animal failed to protect against challenge with the Mexico strain. The animal challenged with the Mexico strain resulted in HEV RNA in the stool and HEV antigen in the liver, although no biochemical or histological evidence of viral hepatitis was observed (Purdy *et al.,* 1993). Similarly, three of four cynos passively immunized with convalescent-phase plasma obtained from a cyno infected with a pool of stool samples containing two Chinese HEV isolates, although protected against disease after challenge with the HEV Pakistan strain SAR-55 with no histological evidence of infection, were still infected, as demonstrated by both viremia and fecal shedding of the virus. However, animals actively immunized with a recombinant 55-kDa SAR-55 ORF2 protein developed neither hepatitis nor viremia when challenged with the SAR-55 strain (Tsarev *et al.,* 1994). Likewise, Pillot *et al.* (1995) showed that infection with one HEV strain may cause protection against disease after challenge with either the homologous or heterologous strain isolated from Asia and Africa, but the protection was limited to preventing clinical disease and not infection because the virus was found in the serum and excreted in the stool. Collectively, these data suggest that although anti-HEV activity contains neutralizing antibody, the immunity elicited as the result of infection or immunization may only protect against developing clinical disease, and not infection. More experimental data are needed before a final conclusion may be drawn regarding the development of an effective HEV vaccine that will protect against both disease and infection.

Cross-neutralization studies in experimentally infected primates using many different HEV strains, although very useful, may be cost prohibitive. Such an extensive study may be best performed using *in vitro* approaches. Recently, a new PCR-based HEV seroneutralization *in vitro* assay was developed (Meng *et al.,* 1997a). This assay was shown to be highly specific and sufficiently sensitive to detect cross-neutralization activity of antibodies against different HEV strains. In this article, we used this approach to study the neutralization of four different HEV strains from Burma, Pakistan, Mexico, and Morocco, representing the four groups of HEV as mentioned above, with anti-HEV-positive serum samples obtained from different sources. The results of this study strongly suggest broad cross-neutralization activity of antibodies elicited during HEV infection. Conversely, immunization of laboratory animals with several recombinant HEV proteins elicited antibodies possessing only a limited range of neutralization activity. Accordingly, recombinant proteins should be carefully chosen as potential HEV vaccine candidates.

RESULTS

PCR detection of HEV RNA of different geographic HEV strains

Full-length nucleotide sequences of 11 different HEV genomes available in GenBank were compared to iden-

tify conserved regions suitable for designing universal PCR primers. A conserved nucleotide region from position 6347–6672 was identified as the most appropriate for designing universal nested PCR primers. A set of four PCR primers was synthesized (see Materials and Methods) and used to amplify with equal efficiency a specific 208-bp PCR fragment from stool specimens from either cynos experimentally infected with the HEV Burma, Mexico, or Pakistan strain or from patients infected with the Morocco strain (data not shown).

Infection of PLC/PRF/5 cells with HEV Burma and Mexico strains

As previously shown (Pillot *et al.,* 1987; Meng *et al.,* 1996, 1997a), the cell line PLC/PRF/5 can be efficiently infected with the HEV Pakistan SAR-55 and Morocco F23 strains. To demonstrate that this cell line is also susceptible to infection with the HEV Burma and Mexico strains, stool suspensions obtained from cynos experimentally infected with the Burma strain or Mexico strain were used to inoculate a monolayer of cells as described previously (Meng *et al.,* 1996). HEV RNA could be continuously detected in the supernatant or in the cells during an incubation period of over 3 weeks (data not shown). Thus, all four inocula used in this study were shown to effectively infect PLC/PRF/5 cells.

Infectivity titration of HEV inocula

To identify end point infectivity titers, serial 10-fold dilutions of each inoculum were prepared and inoculated onto monolayers of PLC/PRF/5 cells. After incubation and washing of the infected cells, RNA was extracted, and HEV RNA was detected by reverse transcription (RT)-PCR as previously described (Meng *et al.,* 1996, 1997a). Infectivity titers for the Burma, Mexico, Pakistan, and Morocco strains were 10^6, 10^8, 10^7, and 10^7 cell culture infectious doses per gram of stool, respectively. Titration experiments were repeated several times to assess infectivity decay over time after storage of filtered stool suspensions at −70°C. No loss of infectivity for any of the four inocula was noted over an observation period of >6 months (data not shown).

Titration of anti-HEV neutralizing activity

Preinoculation and convalescent-phase serum samples obtained from Cy9001, Cy9504, and G21A experimentally infected with HEV Burma, Mexico, and Pakistan strains, respectively, were diluted 2-fold from 1:5 with Hanks' balanced salt solution (HBSS). Approximately 100 cell culture infectious doses of the Burma inoculum were mixed with each dilution of serum specimens collected from the animal infected with the Burma strain. Similarly, the Mexico or Pakistan inoculum was mixed with dilutions of the homologous serum samples. Neutralizing anti-HEV titers were determined by using the rapid PCR-based seroneutralization assay. It was shown that convalescent-phase serum specimens collected 120 days after infection of animals with the Burma or Mexico strain neutralized the infectivity of the homologous Burma or Mexico strains at 1:5, 1:10, and 1:20 dilutions, denoting a neutralization titer of 1:20. For the Pakistan strain, serum samples collected on the 54th day after infection neutralized the infectivity at the 1:40 dilution. In each case, preinoculation serum specimens did not neutralize the homologous virus.

TABLE 1

Cross-Neutralization of the HEV Burma, Mexico, Pakistan, and Morocco Strains with Serum Samples Obtained from Cynomolgus Macaques Experimentally Infected with the Burma, Mexico, and Pakistan Strains

Serum sample	Cross-neutralization with HEV strains			
	Burma	Mexico	Pakistan	Morocco
Cy9001 (Burma; preinoculant)	−−−[a]	−−−	−−	−
Cy9001 (Burma; convalescent, day 120)	+++	−++	++	++
Cy9504 (Mexico; preinoculant)	−−−	−−−	−	−
Cy9504 (Mexico; convalescent, day 120)	−+−+	+−+	++	−+
G21A (Pakistan; preinoculant)	−	−	−	−
G21A (Pakistan; convalescent, day 54)	+++	+++	+	++

[a] Results of replicate assays.

Cross-neutralization of HEV strains with cyno serum

Convalescent-phase serum samples obtained from Cy9001, Cy9504, and G21A as described above were diluted 1:20, 1:20, and 1:40, respectively. Preinoculation serum specimens were diluted 1:10 as negative controls. Each dilution was mixed with ~100 cell culture infectious doses of the Burma, Mexico, Pakistan, and Morocco strains. Neutralization activity was determined by the rapid PCR-based seroneutralization assay. As shown in Table 1, none of the preinoculation serum specimens demonstrated any cross-neutralizing activity. In contrast, all of the convalescent-phase serum samples collected from the animals experimentally infected with the Burma, Mexico, and Pakistan strains demonstrated cross-neutralizing activity for each strain of HEV. Replicates of this experiment are also indicated in Table 1. This experiment confirms that cynos infected with any of the HEV strains used in this study elicit antibodies possessing broad cross-neutralization activity.

Cross-neutralization of HEV strains with human serum obtained from HEV-endemic regions

Eight human convalescent-phase serum samples obtained from individuals residing in Burma (one), India

TABLE 2

Cross-Neutralization of the HEV Burma, Mexico, Pakistan, and Morocco Strains with Human Anti-HEV Positive Serum Specimens Obtained from Different Geographic Regions

Serum sample (1:10)	Cross-neutralization with HEV strains			
	Burma	Mexico	Pakistan	Morocco
Burma	+	+	+	+
India (1)	+++[a]	+−+	++	−+
India (2)	+	+	+	+
China	+++	+−+	++	++
Nepal	+	−+	+	+
Somalia (1)	+	+	+	+
Somalia (2)	+	+	+	+
Mexico	+++	+−+	++	++

[a] Results of replicate assays.

(two), China (one), Nepal (one), Somalia (two), and Mexico (one) were diluted 1:10 with HBSS and mixed with ~100 cell culture infectious doses of either Burma, Mexico, Pakistan, and Morocco strains of HEV. After incubation and inoculation of cell monolayers, the cross-neutralization activity was determined by the rapid PCR-based seroneutralization assay. The results of these experiments (Table 2) confirm that HEV-infected individuals residing in HEV-endemic regions of the world elicit antibodies that cross-neutralize the infectivity of different HEV strains.

Cross-neutralization of HEV strains with human serum obtained from U.S. residents

Eight human convalescent-phase serum samples from U.S. residents with a history of travel to HEV-endemic regions including India, Nepal, Pakistan, Bangladesh, Middle East, and Uruguay before the onset of hepatitis E were diluted 1:10 with HBSS and mixed with the Burma, Mexico, Pakistan, and Morocco strains of HEV, respectively. Following the same experimental protocol that was described above, each convalescent-phase serum cross-neutralized all four HEV strains as shown in Table 3. The results of this neutralization experiment also confirm that HEV-infected individuals who acquired their infection while traveling to an HEV-endemic region of the world elicit antibodies that cross-neutralize the infectivity of different HEV strains.

Cross-neutralization of antibodies against HEV recombinant proteins

Purified IgG obtained by immunization of laboratory animals with three HEV recombinant proteins was used to assess cross-neutralization of different HEV strains. One recombinant protein represented the full-length HEV Burma ORF2-encoded protein expressed in the baculovirus expression system (Mcatee *et al.,* 1996). A second recombinant protein, C2, contained a fragment of the HEV Burma ORF2 protein corresponding to amino acid (aa) position 225–660 (Purdy *et al.,* 1992). A third recombinant protein was an artificial mosaic antigen composed of three regions: (1) 394–470 aa, 562–580 aa, and 631–660 aa from the ORF2-encoded protein of the HEV Burma strain, (2) 91–123 aa from the ORF3-encoded protein of the HEV Mexico strain, and (3) 91–123 aa from the ORF3-encoded protein of the HEV Burma strain (Khudyakov *et al.,* 1994b). Anti-ORF2 was prepared in rabbits, anti-C2 in guinea pigs, and anti-mosaic protein in rabbits and guinea pigs. Serial 2-fold dilutions of each purified IgG preparation were tested against each of the four HEV strains by the seroneutralization assay. As shown in Table 4, both preparations of IgG anti-mosaic protein failed to neutralize any of the HEV strains, whereas the anti-ORF2 antibody neutralized the infectivity of only the Burma and Pakistan strains and only at the lowest

TABLE 3

Cross-Neutralization of the HEV Burma, Mexico, Pakistan, and Morocco Strains with Anti-HEV Positive Serum Specimens Obtained from U.S. Residents Who Had a Travel History before Onset of Disease

Serum sample (1:10)	Travel location	Cross-neutralization with HEV strains			
		Burma	Mexico	Pakistan	Morocco
96-44	India	+	+	+	+
96-58	Asia	+	+	+	+
96-155[a]	Pakistan, India, Nepal	+	+	+	+
96-156[a]	Pakistan, India, Nepal	+	−+[b]	+	+
96-157[a]	Pakistan, India, Nepal	++	++	++	+−+
96-227	Uruguay	+	+	+	−+
97-3	Bangladesh	+	+	+	+
97-36	Middle East	+	+	+	+

[a] Same patient, different dates of serum collection.
[b] Results of replicate assays.

TABLE 4

Neutralizing Effect of Purified IgG Obtained From Animals Immunized with the Full-Length HEV ORF2 Protein, Recombinant C2-Protein, and HEV Mosaic Protein

Dilution of purified IgG	Cross-neutralization with HEV strains			
	Burma	Mexico	Pakistan	Morocco
Anti-ORF2 (from rabbits, 5.5 mg/ml)				
1:10	+	−	+	−
1:20	−	−	−	−
Anti-C2 (from guinea pigs, 3.4 mg/ml)				
1:10	+	+	+	−
1:20	+	+	+	−
1:40	+	+	+	−
1:80	+	+	+	−
1:160	+	+	+	−
1:320	+	+	+	−
1:640	−	−	+	−
1:1280	−	−	−	−
Anti-mosaic protein				
From rabbits, 4.2 mg/ml, 1:10	−	−	−	−
From guinea pigs, 2.4 mg/ml, 1:10	−	−	−	−

dilution tested. No neutralization activity was observed against the Mexico and Morocco strains. However, IgG anti-C2 demonstrated efficient neutralization of not only the HEV Burma and Pakistan strains but also the Mexico strain, despite having a lower IgG concentration compared with the IgG anti-ORF2 preparation (Materials and Methods). Surprisingly, the infectivity of the HEV Morocco strain was not neutralized by any of the IgG preparations at any of the tested dilutions. Because a pool of fecal specimens collected from 15 different individuals from one Morocco HEV outbreak (see Materials and Methods) was used, a possible explanation for the absence of neutralization may be the existence of one or more neutralization resistant variants in the pool. To examine this possibility, an HEV PCR fragment obtained from the Morocco inoculum and from inoculated cells before and after neutralization was sequenced and compared. This comparison demonstrated complete identity between each fragment (data not shown). This finding suggests that despite the fact that the Morocco inoculum was composed of a pool of 15 fecal specimens, the HEV adsorbed onto the surface of inoculated cells is predominant in this inoculum and therefore is representative of the epidemic HEV strain. In addition, the absence of any nucleotide changes identified within these sequences suggests that the HEV population in the Morocco inoculum is homogeneous. Thus, in contrast to the observation made for antibodies elicited during a natural HEV infection, only limited cross-neutralization activity was observed for antibodies elicited against ORF2 recombinant protein.

DISCUSSION

The rapid PCR-based HEV seroneutralization assay previously described (Meng *et al.,* 1997a) was used in the present study to demonstrate that this assay is a very reliable and highly reproducible *in vitro* approach to evaluate the neutralizing activity found in various anti-HEV serum specimens and that the method represents a very efficient and fast alternative to *in vivo* animal models. As shown previously (Pillot *et al.,* 1987; Meng *et al.,* 1996, 1997a) and again in this study, PLC/PRF/5 cells are susceptible to infection with four different HEV strains. Although no cytopathic effect appeared, HEV RNA could be detected in HEV-inoculated cells for >3 weeks despite extensive cell washing and a change of media every 2–3 days. The detection of HEV RNA confirms that this system satisfies at least the first step of the infection process, namely, adsorption onto the surface of PLC/PRF/5 cells. As a result, this system allows for the development of a rapid *in vitro* seroneutralization assay to evaluate the neutralizing activity of anti-HEV positive serum specimens by blocking the adsorption process onto the cell surface. A very similar system was developed to study the neutralizing antibody response to the hepatitis C virus, a virus that remains refractory to propagation in cell culture (Shimizu *et al.,* 1994, 1996; Zibert *et al.,* 1997). These assays are based on using cell surface receptors to specifically capture the virus, followed by RT-PCR to efficiently detect the genome of the captured virus. Using this system, antibody neutralization activity may be assessed by demonstrating the absence of detectable virus genome in inoculated cells. In the absence of a reliable permissive cell culture system, the assay may be used as a rapid alternative to primate models to evaluate various recombinant proteins as potential vaccine candidates.

The results presented in this study confirm that convalescent-phase antibodies elicited as the result of infection with one HEV strain can efficiently block adsorption of several HEV strains onto the surface of PLC/PRF/5 cells. This observation suggests that convalescent-phase antibody can cross-neutralize the infectivity of multiple HEV strains. Specifically, serum samples obtained from cynos experimentally infected with the HEV Burma, Mexico, or Pakistan strain neutralized the infectivity of four HEV strains from Burma, Mexico, Pakistan, and Morocco. Moreover, serum samples obtained from individuals residing in HEV-endemic regions of the world including Burma, India, China, Nepal, Somalia, and Mexico, or obtained from U.S. residents with a history of travel to HEV-endemic regions (including India, Pakistan, Nepal, Uruguay, Bangladesh, and Middle East) before the onset of the disease also neutralized the infectivity of all four strains *in vitro.* Although very reproducible, especially when homologous virus and antibodies are used, the HEV seroneutralization *in vitro* assay, when

applied to Mexico and Morocco strains and serum samples of Asian origin, resulted in several inconsistent results in replicate assays (Tables 1, 2, and 3). Similar inconsistencies were observed previously (Meng *et al.,* 1997a) in which convalescent-phase serum specimens obtained from a cyno experimentally infected with an Algerian HEV isolate neutralized the infectivity of the Pakistan strain (SAR-55) failed to definitively neutralize the Morocco F23 strain. These observations may be explained, at least in part, by variability in the efficiency of neutralization during the short incubation period, by low-titer anti-HEV neutralizing antibodies, or by both. Alternatively, some fine antigenic heterogeneity that may play a role in the neutralization activity may exist as the result of aa sequence heterogeneity between different HEV strains, as exemplified by a 7% sequence heterogeneity between the ORF2-encoded proteins of the HEV strains from Mexico and Asia (Yin *et al.,* 1994). In addition, sequence heterogeneity between the ORF3-encoded proteins of the HEV Burma and Mexico strains was shown to influence the antigenic heterogeneity of this protein (Yarbough *et al.,* 1991; Khudyakov *et al.,* 1993, 1994a). Nevertheless, no strong evidence exists suggesting that sequence and antigenic variations have any effect on the HEV neutralizing antigenic epitope or epitopes. A thorough evaluation of the antigenic diversity of the ORF2 protein from different HEV isolates should be established before any final conclusions are drawn on the possible diversity of the HEV neutralization epitope or epitopes and the neutralizing activity of anti-HEV antibodies specific to different HEV strains.

The results obtained in this study with antibodies against various HEV recombinant antigens provide additional evidence of the possible heterogeneity of the neutralizing antigenic epitope or epitopes. Antibodies against the recombinant proteins of the HEV Burma strain effectively neutralized the HEV strains from Burma and Pakistan but showed no neutralizing activity against the HEV Morocco strain. It should be noted, however, that the HEV neutralizing antigenic epitope or epitopes may be modeled with recombinant proteins differently from the native epitope or epitopes. In addition, it is conceivable that different recombinant proteins can model antigenic epitopes with different efficiencies. In agreement with this assumption, we found that antibodies against the recombinant protein C2, which contains only a part of the ORF2-encoded protein (Purdy *et al.,* 1992), could neutralize the infectivity of the HEV Mexico strain, whereas antibodies against the full-length ORF2-encoded protein (Mcatee *et al.,* 1996) failed to neutralize the infectivity of the same strain. Thus, antibodies to a shorter fragment of the HEV ORF2 protein were shown to have more efficient neutralizing activity than the full-length protein. The difference between the antigenic properties of a fragment and the full-length HEV ORF2-encoded protein was recently observed for the detection of convalescent-phase anti-HEV using different HEV recombinant proteins (Li *et al.,* 1994, 1997). These experiments revealed that full-length ORF2 protein was poorly reactive with convalescent-phase serum samples, whereas the C-terminal portion of the ORF2 protein was highly reactive.

In conclusion, the data presented in this study suggest that antibodies elicited during an HEV infection display broad HEV-neutralizing activity, whereas antibodies elicited after immunization with recombinant Burma ORF2 proteins demonstrate a more restricted ability to neutralize various HEV strains obtained from different HEV-endemic region of the world.

MATERIALS AND METHODS

Cell culture

PLC/PRF/5, a human hepatocarcinoma cell line, was grown in Dulbecco's modified Eagle's medium (GIBCO BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratory, Logan, UT), and incubated at 37°C with 5% CO_2. For virus titration and for the neutralization assay, trypsinized cells were seeded onto 24-well, flat-bottom culture plates at a concentration of 10^5 cells per well.

Virus stocks

The HEV Burma strain was obtained from a fecal specimen collected 17 days after experimental infection of a cyno (Cy9210) inoculated with a stool pool from a third-passage Burma strain-infected cyno (Cy73) (Bradley *et al.,* 1987). The HEV Mexico strain was from a fecal specimen collected 21 days after experimental infection of a cyno (Cy9218) inoculated with a stool suspension from a patient (Mex 14) with hepatitis E during an outbreak in Telixtac, Mexico. Mex 14 was shown previously to cause hepatitis E in all experimentally infected cynos and chimpanzees (Bradley *et al.,* 1988; Bradley, 1990). Both Burma and Mexico strains were kindly provided by K. McCaustland [Hepatitis Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA]. The HEV Pakistan strain, SAR-55 (Tsarev *et al.,* 1992), a gift provided by Dr. R. H. Purcell (Hepatitis Viruses Section, National Institutes of Health, Bethesda, MD), was obtained from a fecal specimen of an experimentally infected rhesus monkey 21 days after intravenous inoculation (Pillot *et al.,* 1995). The HEV Morocco strain was obtained from a fecal pool of 15 patients with well-documented epidemic hepatitis E who were residing in the area of Casablanca, Morocco (Benjelloun et al., 1997). Each fecal specimen was prepared as a 10% (wt/vol) suspension in PBS, filtered through a Sterilization Filter Unit (Nalge Company, Rochester, NY), and stored at −70°C.

Serum samples

Burma anti-HEV serum was collected from a cyno (Cy9001) 120 days after inoculation with the HEV

Burma inoculum obtained from Cy73. Mexico anti-HEV serum was collected from a cyno (Cy9504) 120 days after inoculation with the HEV Mex 14 inoculum. These two serum specimens were kindly provided by J. Spelbring (Hepatitis Branch, CDC). Pakistan anti-HEV serum was collected from a cyno (G21A) 54 days after inoculation with SAR-55. Preinoculation serum samples were obtained from each animal before experimental infection. Eight human serum specimens (kindly provided by Dr. M. Favorov and K. McCaustland, Hepatitis Branch, CDC) were obtained from convalescent patients from different geographic regions (Burma, India, China, Nepal, Somalia, and Mexico). Another eight convalescent-phase human serum specimens (kindly provided by S. Lambert and Dr. E. Mast, Hepatitis Branch, CDC) were obtained from individuals residing in the United States. All eight individuals reported a travel history to India, Nepal, Pakistan, Bangladesh, Middle East, or Uruguay and returned to the United States within the expected incubation period before the onset of disease. All of the preinoculation and convalescent-phase serum samples were inactivated at 56°C for 30 min. Only the convalescent-phase serum specimens were shown to contain anti-HEV activity by enzyme immunoassay using both an HEV mosaic antigen (Khudyakov *et al.,* 1994b) and a commercially available kit (Genelabs Diagnostics, Singapore).

Purified IgG

Anti-ORF2 IgG, at a concentration of 5.5 mg/ml, was prepared from serum specimens of rabbits immunized with a full-length Burma strain ORF2-encoded, 73-kDa protein expressed in insect cells using a recombinant baculovirus (Mcatee *et al.,* 1996). Anti-C2 IgG, at a concentration of 3.4 mg/ml, was prepared from serum samples of guinea pigs immunized with a trpE-HEV fusion protein (C2 protein) that represents the carboxyl two thirds of the putative capsid protein of the Burma strain and expressed in *Escherichia coli* with a pATH expression system (Purdy *et al.,* 1992). Both IgG anti-ORF2 and anti-C2 were kindly provided by Dr. M. Purdy (Hepatitis Branch, CDC). IgG anti-mosaic protein, at a concentration of 4.2 mg/ml, was prepared from serum samples of rabbits immunized with an HEV-specific artificial mosaic protein consisting of a series of short (10–22 aa) linear antigenic epitopes from ORF2 and ORF3 of both the Burma and Mexico strains and expressed in *E. coli* as a chimera with glutathione-*S*-transferase (Khudyakov *et al.,* 1994b). Another IgG anti-mosaic protein, at a concentration of 2.4 mg/ml, was prepared from serum specimens of guinea pigs immunized with the same HEV mosaic protein. Both IgG anti-mosaic proteins were kindly provided by Dr. M. Favorov (Hepatitis Branch, CDC).

Rapid PCR-based seroneutralization assay in cell culture

This protocol has been previously described (Meng *et al.,* 1997a), except for the PCR primers used in this study. Briefly, ~100 cell culture infectious doses of an HEV inoculum diluted in 100 μl of HBSS were mixed with 100 μl of a serum sample at an appropriate dilution. After incubation at 37°C for 1 h, the mixture was inoculated onto a cell monolayer of PLC/PRF/5. After adsorption for 2 h at 37°C, the cells were washed three times with HBSS followed by immediate RNA extraction with TRIzol reagent (GIBCO BRL) according to the manufacturer's instructions. RT-nested PCR was performed by using a set of universal HEV PCR primers. The outer primers were YK-1291 (5′-GTT GTC TCA GCC AAT GGC GAG CC) and YK-1294 (5′-GCC TGC GCG CCG GTC GCA ACA), and the inner primers were YK-1292 (5′-TGG AGA ATG CTC AGC AGG ATA A) and YK-1293 (5′-TAA GTG GAC TGG TCG TAC TCG GC). Both the first- and second-round amplifications were carried out according to the following cycling program: denaturation at 94°C for 45 s, annealing at 60°C for 20 s, and extension at 72°C for 60 s and for 30 cycles. Amplicons were separated by agarose gel electrophoresis with size markers and visualized by ethidium bromide fluorescence. Neutralization was determined by the absence of detectable HEV RNA in the inoculated cell culture. A negative serum control, virus control, and uninoculated cell control were always processed for the detection of HEV RNA at the same time for each assay.

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